Lack of Distinction Between *Nitrobacter agilis* and *Nitrobacter winogradskyi*

**PAULINE H. C. PAN**

Department of Bacteriology, Rutgers—The State University, New Brunswick, New Jersey 08903

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No adequate criteria were established to distinguish between *Nitrobacter agilis* and *N. winogradskyi*. However, very gentle preparative techniques permitted demonstration of flagella in *N. agilis*.

The principal distinction between *Nitrobacter agilis* and *N. winogradskyi* has been the motility of the former. Yet even this criterion is not constant. Of eight reports surveyed by Zavarzin and Legunkova (20), two reported *N. winogradskyi* to be motile (during the log phase), and of three reports on *N. agilis*, one reported it to be non-motile. During the course of studies on *N. agilis*, we have found that the flagella are exceedingly fragile, and simple preparative manipulations (pipetting, centrifuging, scraping colonies off agar with a glass rod, etc.) cause the disappearance of flagella, whereas very gentle preparative techniques of collecting the cells directly from the colonies with the electron microscope grid permitted their demonstration.

The culture of *N. agilis* used was isolated by H. L. Jensen and was obtained from the laboratory of D. Pramer. It was cultivated on the medium of Lewis and Pramer (10) supplemented with 150 μg of biotin per liter (9) and 250 μg of NaMoO₄ per liter (6). Since growth in fluid media was not helpful for electron microscopy, 1.5% ion agar was used as the solidifying agent. The surface colonies, after 7 to 9 days of incubation at 26 °C, were gently touched with hydrophilic carbon-coated copper grids (7) and stained with 1% phosphotungstic acid at pH 7.2 for 4 min and observed in a Jem 120 electron microscope at instrumental magnifications of 14,000 and 64,000× according to techniques described elsewhere (7).

Motility of live unstained preparations of *N. agilis* could be observed only in dense cell populations, and electron micrographs of cells prepared in the usual way showed no flagella. Figure 1A shows a flagellated cell with the flagella perhaps three times the length of the cell. Figure 1B shows a characteristic clump of eight cells, with no flagella obtained by scraping colonies off the agar surface rather than merely touching the grid to them (as done in Fig. 1A and 1C). Figure 1C shows a similar cluster with flagella. The form of the cell and other properties are not inconsistent with previously published photographs of *Nitrobacter* (12-14, 20). The wrinkled surface (demonstrable also by shadowing and uranyl acetate staining) seems reasonably typical of certain other gram-negative cells (3, 8, 19), but the cleftlike invaginations (especially in Fig. 1B and 1C) may be artifacts produced during manipulations such as staining; in our hands, the same methods do not produce such artifacts with other gram-negative organisms.

Search through many photographs has never revealed basal plates to the flagella such as those reported for *Rhodospirillum rubrum* (4) and *Bacillus stearothermophilus* (2). Rather, the attachment seems to be simpler, perhaps resembling that reported for *B. pumilus* (1). Figure 1A suggests a polar flagellum, but we find a predominance of randomly distributed forms (as in Fig. 1C). Literature reports claim a single polar flagellum for *N. agilis* (13) and a single lateral flagellum for the alleged nonmotile *N. winogradskyi* (17). An almost parallel situation occurs in *Nitrosomonas. N. europae* has been reported as both motile and nonmotile (11, 18), whereas the electron micrographs show flagellation (5, 15). It seems evident that flagellation is not a good criterion in either of these genera.

A further possible distinction might be found in the report of Smith and Hoare (16) that *N. agilis* is stimulated by the addition of acetate and that, when properly supplemented, it will grow on acetate as its energy source. The situation with respect to *N. winogradskyi* is not known. However, we have been unable to grow our strain of *N. agilis* on acetate. (The strain used by Smith and Hoare appears to be no longer available.) Figure 2 shows the data reported by Smith and Hoare (dashed lines) together with our own.

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1 Present address: Biology Department, Princeton University, Princeton, N.J. 08540.
Fig. 1. Influence of sample preparation on flagella. (A) An example of single flagellum, $3.6 \times 10^3$ nm long. Nitrobacter agilis cells obtained by picking up colonies from agar surface with copper grid. Phosphotungstate in all. (B) Characteristic cluster of eight cells without flagella obtained by scraping cells off of agar surface. (C) Randomly attached flagella, 10 to 12 nm in diameter, obtained by picking up colony from agar surface. Magnification bar represents 0.1 μm.
data (solid lines). Further experiments (Table 1) showed that acetate addition to nitrite medium does not enhance growth nor was growth on acetate itself possible with either the minimal salts medium described by Smith and Hoare (SH medium) (16) or in the more complex casein hydrolysate medium with nitrite, in each case, replaced by acetate (5 mmole/100 ml). Eventually, not all strains of N. agilis respond to acetate. Our strain, however, is capable of growing on glucose in the absence of nitrite, provided that flowthrough dialysis conditions are met.

It seems evident that there are at present no adequate criteria to distinguish between N. agilis and N. winogradskyi.

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**LITERATURE CITED**


**FIG. 2. Growth of Nitrobacter agilis on nitrite and acetate.** Data from Smith and Hoare (16) show growth on nitrite alone, control (O-O-O); supplemented with acetate (5 mm) at inoculation (O-O-O), and after 190 hr (O-O-O). Our data show growth on nitrite alone, controls (SN medium, O-O-O, and SH medium, A-A-A); supplemented with acetate (5 mm) after 370 hr (O-O-O) and grown with acetate alone (SH medium, A-A-A, and SN medium, O-O-O). Arrows indicate completion of nitrite oxidation.

**TABLE 1. Growth of Nitrobacter agilis on acetate and nitrite**

<table>
<thead>
<tr>
<th>Growth medium*</th>
<th>Growth (μg of protein/ml) at 200 hr</th>
<th>400 hr</th>
<th>600 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite SN medium</td>
<td>15.3</td>
<td>13.2</td>
<td>14.1</td>
</tr>
<tr>
<td>Nitrite SH medium</td>
<td>16.2</td>
<td>15.8</td>
<td>13.4</td>
</tr>
<tr>
<td>Nitrite SN medium</td>
<td>14.8</td>
<td>15.1</td>
<td>13.3</td>
</tr>
<tr>
<td>Nitrite SH medium</td>
<td>16.1</td>
<td>14.9</td>
<td>12.9</td>
</tr>
<tr>
<td>Nitrite + acetate (5 mM) SN medium</td>
<td>16.2</td>
<td>18.2</td>
<td>19.4</td>
</tr>
<tr>
<td>Nitrite + acetate (5 mM) SH medium</td>
<td>17.1</td>
<td>18.8</td>
<td>19.6</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (0.1%) + acetate (5 mM) SN medium</td>
<td>3.5</td>
<td>5.7</td>
<td>6.9</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (0.1%) + acetate (5 mM) SH medium</td>
<td>3.4</td>
<td>5.2</td>
<td>4.8</td>
</tr>
<tr>
<td>Casein hydrolysate (0.5%) + acetate (5 mM) SN medium</td>
<td>2.4</td>
<td>3.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Casein hydrolysate (0.5%) + acetate (5 mM) SH medium</td>
<td>2.3</td>
<td>2.8</td>
<td>3.9</td>
</tr>
</tbody>
</table>

*SN medium, base medium of Lewis and Pramer (10) with biotin and molybdenum. SH medium, base medium of Smith and Hoare (16).

*Acetate added after NO₃ exhausted, 320 hr.